Sains Malaysiana 52(3)(2023): 821-835
http://doi.org/10.17576/jsm-2023-5203-11

Molecular Characterization of Ochratoxin a Producing Indigenous *Aspergillus* Strains from Poultry Feed in Pakistan

(Pencirian Molekul Okratoksin Penghasil Strain *Aspergillus* Asli daripada Makanan Poltri di Pakistan)

GULL NAZ[1], AFTAB AHMAD ANJUM[1*], TEHREEM ALI[1], MUHAMMAD NAWAZ[1], SANAULLAH IQBAL[2], MATEEN ABBAS[3] & RABIA MANZOOR[1]

[1]*Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[2]*Department of Food Science and Human Nutrition, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[3]*Quality Operations Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan*

Received: 12 July 2022/Accepted: 17 January 2023

ABSTRACT

Ochratoxin A (OTA) is nephrocarcinogenic and immunosuppressive toxin and OTA producing molds contaminate the food crops. Isolation and identification of ochratoxin producing fungi was carried out from poultry feed samples (n=120) followed by preliminary confirmation through macroscopic and microscopic characteristics. Purified fungal isolates identified as *Aspergillus* 1842(91.68%) followed by *Penicillium* 91 (4.53%), *Mucor* 52 (2.58), *Alternaria* 7 (0.35%), *Cladosporium* 6 (0.29%), *Fusarium* 4 (0.199%) and unidentified (07). OTA production was confirmed through thin layer chromatography (TLC) followed by high performance liquid chromatography (HPLC). Only 41 isolates (2.22%) out of 1842 *Aspergillus* isolates were able to produce toxin. At genetic level, characterization was performed through polymerase chain reaction (PCR) using species specific gene primers. From 41 isolates 27, 9 and 5 were characterized as *Aspergillus terreus*, *Aspergillus parasiticus,* and *Aspergillus ochraceus,* respectively. Physical and chemical factors were optimized for OTA production. Under the effect of 37 °C temperature and 7.5 pH of Sabouraud dextrose broth (SDB) medium, higher toxin (969.45±.03 µg/mL) production was observed from ASPO-6 isolate. ASPO-4 isolate produce higher toxin amount in SDB medium with supplementation of maize 5%, wheat 1% and rice 3%. OTA stability was determined by adjusting standard concentration of 100 µg/mL in organic solvents (chloroform, acetonitrile and methanol) and organic solids. Least percentage log reduction in OTA concentration and stability of OTA was observed in opaque vials with chloroform and sucrose and transparent vials with sucrose after 6 months. OTA can be used as indigenous standard for identification of OTA from field samples.

Keywords: *Aspergillus*; Ochratoxin A; polymerase chain reaction; stability; thin layer chromatography

ABSTRAK

Okratoksin A (OTA) ialah toksin nefrokarsinogenik dan imunosupresif dan kulapuk penghasilan OTA mencemari tanaman makanan. Pengasingan dan pengenalpastian kulat penghasilan okratoksin telah dijalankan daripada sampel makanan poltri (n=120) diikuti dengan pengesahan awal melalui ciri makroskopik dan mikroskopik. Pengasingan kulat tulen dikenal pasti sebagai *Aspergillus* 1842(91.68%) diikuti oleh *Penicillium* 91(4.53%), *Mucor* 52(2.58), *Alternaria* 7(0.35%), *Cladosporium* 6(0.29%), *Fusarium* 4(0.199%) dan tidak dikenal pasti (07). Pengeluaran OTA telah disahkan melalui kromatografi lapisan nipis (TLC) diikuti oleh kromatografi cecair prestasi tinggi (HPLC). Hanya 41 pencilan (2.22%) daripada 1842 pencilan *Aspergillus* dapat menghasilkan toksin. Pada peringkat genetik, pencirian dilakukan melalui tindak balas rantai polimerase (PCR) menggunakan primer gen khusus spesies. Daripada 41 pencilan 27, 9 dan 5 masing-masing dicirikan sebagai *Aspergillus terreus*, *Aspergillus parasiticus* dan *Aspergillus ochraceus*. Faktor fizikal dan kimia telah dioptimumkan untuk pengeluaran OTA. Di bawah kesan suhu 37 °C dan 7.5 pH medium *Sabouraud dextrose broth* (SDB), pengeluaran toksin yang lebih tinggi (969.45±.03 µg/mL) diperhatikan daripada pencilan ASPO-6. Pengasingan ASPO-4 menghasilkan jumlah toksin yang lebih tinggi dalam medium SDB

dengan tambahan jagung 5%, gandum 1% dan beras 3%. Kestabilan OTA ditentukan dengan melaraskan kepekatan piawai 100 µg/mL dalam pelarut organik (kloroform, asetonitril dan metanol) dan pepejal organik. Peratusan log pengurangan paling sedikit dalam kepekatan OTA dan kestabilan OTA diperhatikan dalam botol legap dengan kloroform dan sukrosa dan botol lutsinar dengan sukrosa selepas 6 bulan. OTA boleh digunakan sebagai piawaian asli untuk mengenal pasti OTA daripada sampel lapangan.

Kata kunci: *Aspergillus*; kestabilan; kromatografi lapisan nipis; okratoksin A; tindak balas rantai polimerase

Introduction

Several diseases of poultry, animals and human are caused by consumption of feed or food contaminated by microorganisms or their toxic metabolites (Koh et al. 2018). Various toxigenic fungal species contaminate agricultural products and produce toxins called mycotoxins. Poultry feed contamination by these mycotoxins is a major issue in poultry production effecting health as well as performance and also danger to human health by consumption of contaminated poultry meat (Zeng et al. 2019). Commonly used poultry feed mixtures contain grains as a source of carbohydrates and mycotoxin producing fungi are main grain contaminants (Pleadin 2015). The important mycotoxin producing fungi are *Aspergillus*, *Fusarium* and *Penicillium* contaminating the feed stuff at pre and post harvesting stages as well as during storage (Sivakumar, Singaravelu & Sivamani 2014). *Penicillium*, *Aspergillus* and *Cladosporium* are called storage fungi because of their ability to contaminate feed and feed ingredients during storage (Maciorowski et al. 2007).

According to Food and Agriculture Organization (FAO), 25% of the crops get contaminated by molds and their toxins and cause economic losses (Trail et al. 1995). Important factors contribute to fungal growth and mycotoxin production are poor harvesting practices, improper drying, handling, packaging, storage, and transport conditions (Bhat, Rai & Karim 2010). Both zones (tropical and subtropical) are observed in Pakistan, therefore, high rain fall and strong sunshine is also included in its climate. This kind of climate is very promising fungal growth especially *Aspergillus* which is thermotolerant species (Hendrix et al. 2018). Mycotoxins contamination of food stuffs and crop spoilage are the targets of these species which are known as fungal contaminants over the Asia (Ehrlich et al. 2007). The presence of mycotoxins in poultry feed is a major problem and cause economic loss to poultry sector in Pakistan (Rafique, Javed & Ali 2018).

OTA is nephrotoxic, nephrocarcinogenic and immunosuppressive toxin; a fungal secondary metabolite produced mainly by *Aspergillus* species (Petzinger & Weidenbach 2002; Rosa et al. 2002). Various *Aspergillus* species such as *A. ochraceus***,** *A. terreus*, *A. parasiticus Aspergillus niger, Aspergillus melleus,* and *Aspergillus carbonarious* produce OTA in hot and humid climate while *Penicillium* species such as *Penicillium verrucosum* in cold or temperate environments (Antonio-Bautista et al. 2021). These species contaminate approximately a quarter of food crops and cause economic losses by producing their toxic metabolites particularly OTA (Cervini et al. 2021). Normally OTA concentration does not exceed few ppb but higher values also detected in various foodstuffs by using immunoaffinity columns (Mantle 2002). OTA is one of the mycotoxins that have aroused public distress over the world due to its renal toxicity and molecular characterization of fungal species producing OTA has its own significance. OTA associated with the pathogenesis of various kinds of human nephropathies especially kidney cancer is shown by various epidemiological studies (Illueca et al. 2021). World Health Organization (WHO) and International Agency for Research on Cancer has classified OTA as a possible human carcinogen (Group 2B) (IARC 1993). Present study has been designed for molecular characterization of OTA producing fungi such as *A. ochraceus, A. terreus,* and *A. parasiticus* isolated from stored rice, maize and wheat samples collected from district Lahore, Punjab, Pakistan as well as physicochemical optimization for OTA production.

Materials and Methods

Sampling

Poultry feed samples including home mixed poultry feed, commercial mashed and pelleted poultry feed and feed ingredients such as stored rice, maize and wheat were collected from District Lahore following the method of Saleemi et al. (2010) with minor modifications. A total of 120 samples (n=20, each) were collected in properly labelled sterile polythene bags. Random sampling of 3 kg

feed was done from three different sites of a feed lot and pooled together and 1 kg was transported to Mycology Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.

SAMPLE PROCESSING, ISOLATION AND PURIFICATION

Samples were triturated to fine powder and 10 g of each sample was added in 250 mL blue capped sterile bottle containing 90 mL sterilized peptone water (0.1%). Fungal count was determined in one g of each selected sample. Ten-fold serial dilution (10^{-1} to 10^{-10}) of each sample was prepared in sterilized peptone water present in glass test tubes (09 mL each) (Saddozai, Raza & Saleem 2012). All of the experimental work was carried out aseptically in safety cabinet (BSC Class 2 B2). From each dilution 100 µL was inoculated on Sabouraud Dextrose agar medium plates by spreading technique as described by Kehinde et al. (2014). The inoculated medium plates were incubated at 25±3°C for three to five days and observed daily for fungal growth. From Mixed fungal growth on SDA medium plates, single fungal isolates colonies were purified by secondary culturing on the SDA medium plates. The inoculated plates were incubated at 25±3°C for two to three days and observed daily for purified cultures of fungi. Purified fungal colonies were photographed from both sides (obverse and reverse) at immature and mature stages to record macroscopic characters.

IDENTIFICATION OF FUNGAL ISOLATES

Different types of fungi were identified from selected samples based on their macroscopic (obverse and reverse side of culture on medium plates) and microscopic characters (cellophane tape, wet mount and drop method of fungal slide culture) by following guidelines for specific genus and species (Becker et al. 2019). Distribution of fungi and relative density of genus and specie were also determined following the method of Greco et al. (2014) with minor modifications.

CONFIRMATION OF OTA PRODUCING *ASPERGILLUS* SPECIES

Toxin producing isolates of different *Aspergillus* species were confirmed by PCR. *A. ochraceus*, *A. terreus,* and *A. parasiticus* were confirmed by amplification of Specie specific region of genome by PCR (Dudakova et al. 2017).

DNA EXTRACTION AND ESTIMATION

Three days old fungal mycelia in broth culture were used for DNA extraction by plant DNA extraction kit (Exgene Plant SV mini, 100p, Cat no. 117-101) following the manufacturer instructions.

MOLECULAR CONFIRMATION BY PCR

PCR was carried out following the procedure described by Henry et al. (2000) targeting internal transcribed regions of rRNA using three sets of primers: **OCR1/OCR2** (5′-CTTCCTTAGGGGTGGCACAGC-3′ and 5′-GTTGCTTTTCAGCGTCGGCC-3′, respectively) for *A. ochraceus*; **TER1/TER2** (5′-GTAGGGTTCCTAGCGAACC-3′ and 5′-GGAAAAAGATTGATTTGCGTTC-3′, respectively) for *A. terreus* and **PAR1/PAR2** (5′-GTCATGGCCGCCGGGGGCGTC-3′ and 5′-CCTGGAAAAAATGGTTGTTTTGCG3′, respectively) for *A. parasiticus* designed by Primer3 software. Reaction mixture of 25 µL for each isolate was prepared in the PCR tube (0.2 mL). Components of the mixture are; DNA (03 µL), forward primer (1.25 µL), reverse primer (1.25 µL), master mix (12.5 µL) and distilled water (7 µL). Reaction mixture was placed in thermocycler (ICCC/PTC-06) and conditions for *A. ochraceus* were as follows; initial denaturation at 95 ºC 04 min 30 s, 30 cycles of 30 s at 95 ºC (denaturation), 30 s at 63, 58 and 69.3 ºC (annealing for *A. ochraceus, A. terreus,* and *A. parasiticus,* respectively), 01 min at 72 ºC (extension) and finally 03 min at 72 ºC. Amplicons were detected at 1.8% agarose gel electrophoresis and visualized under UV light.

SCREENING FOR OTA PRODUCING *ASPERGILLUS* spp.

Different *Aspergillus* species recovered from feed samples were screened for OTA production by following method described by Ren et al. (1999). TLC was used to detect OTA producing fungi.

PRODUCTION OF OTA

Standard spore inoculum (approximately 10^6 spores/mL) of each *Aspergillus* species pure cultures was prepared in normal saline (Gaur & Adholeya 2000). In 100 mL SDB medium 10% v/v of each purified *Aspergillus* isolate inoculum was added. All flasks were properly wrapped with brown paper, labelled and placed in incubator (25±3 ºC) for up to 45 days with off and on shaking.

After 45 days' fungal broth cultures were autoclaved at 121 ºC under pressure of 15 psi for 15 min and used for OTA extraction. Autoclaved fungal cultures were homogenized with a tissue homogenizer. Extraction

mixture was prepared by adding homogenized fungal culture (25 g), dichloromethane (125 mL) and diluted phosphoric acid (12.5 mL) into a conical glass flask (250 mL). Mixture was placed in orbital shaker for continuous shaking of at least one hour. Mixture was passed through Whatman filter paper no 42 (pore size is 2.5 µm). The filtrates were evaporated to dry by placing in water bath at 50 °C. Filtrates were reconstituted in chloroform (03 mL), mixed and allowed to stand for at least five min to settle down solids. Liquid phase having OTA was collected with sterile glass pipette and poured into test tube. Reconstitution of filtrate with chloroform was repeated for three times to obtain maximum amount of toxin. Chloroform was again evaporated till 1 mL volume (Hussaini et al. 2009).

DETECTION OF OTA BY TLC

Toxin recovered was detected qualitatively by TLC on silica gel coated aluminum sheets (20 × 20 cm). The sheets were activated by placing in hot air oven at 50 ºC for 1-2 min to remove moisture. Samples were spotted (30 µL each) with a distance of one cm from each other on bottom line at TLC sheets, air dried and labeled properly. A standard solution of OTA was spotted (10 µL) along with samples. TLC sheets were placed vertically in TLC tank having mobile phase (toluene: methanol: acetic acid – 90:5:5). Mobile phase was allowed to run up to line drawn at top under controlled room temperature (25 ºC). Sheets were removed from developing tank, air died and viewed in UV illuminator at 365 nm. Samples were considered positive for OTA matching with standard in terms of fluorescence and photographed using camera.

PHYSICOCHEMICAL OPTIMIZATION FOR OTA PRODUCTION

Different OTA producing *Aspergillus* species confirmed by PCR were optimized for highest OTA production using different physical (temperature 22, 28 and 37 ºC) and initial pH of medium (4.5, 06, and 7.5) and chemical (substrates at different concentrations rice, maize and wheat grains (1, 3 and 5%) parameters. Standard spore inoculum (~10^6 spores/mL) was prepared from each *Aspergillus* pure culture. SDB inoculated with 10% v/v spore suspension, incubated for at least 30 days and observation recorded (Pardo et al. 2005). Fungal cultures were autoclaved and processed for OTA extraction as described above. Extracted toxin was quantified by HPLC and production potential at different pH, temperatures and substrates was recorded.

QUANTIFICATION OF OTA

OTA produced by selected fungal isolates under different conditions was quantified by HPLC along with known OTA standard following the procedure of Pena et al. (2005). Preliminary detection of OTA was completed by TLC (mentioned above) and then further quantified by HPLC. Toxin samples were derivatized prior to run in HPLC. Extracted samples (02 mL) dissolved in acetonitrile were evaporated and reconstituted in a mixture of Trifluoroacetic acid (50 µL) and n-hexane (200 µL). Mixture was vortexed and placed for five min at room temperature (25 ºC). Acetonitrile solution (1950 µL) prepared by adding one part of acetonitrile in nine parts of distilled water was added in the mixture post incubation. Vortex mixture was filtered through filter paper (0.25 µm) and poured in a brown vial ready to use in HPLC system. Mobile phase of HPLC was prepared by adding acetonitrile, water and acetic acid in ratio of 49.5:49.5:1.0 v/v/v respectively, loaded in HPLC system and allowed to run for at least 30 to 40 min. Mobile phase was maintained at a flow rate of 1 mL per min and maximum pressure 400 bar was adjusted. Temperature provided to C18 columns was 28 ºC. A standard solution of OTA (10 µg/mL) was injected to the system followed by sample. An excitation wavelength was 333 nm and emission wavelength were 460 nm in detection system. For both excitation as well as emission, spectral bandwidth was 10 nm. Peaks of standard as well as samples were appeared on chromatograms.

STATISTICAL ANALYSIS

Data obtain from OTA quantification by HPLC was statistically analyzed by Duncan's multiple range test using statistical package for social sciences (SPSS) version 20.0.

RESULTS

POULTRY FEED MYCOFLORA

Mycoflora in poultry feed and feed ingredients (n=120) was determined on the basis of macroscopic and microscopic characters of pure cultures of fungi 2009 isolates of different genera including *Aspergillus*, *Penicillium, Mucor, Alternaria, Cladosporium* and *Fusarium*. Preliminary identification of isolated fungi along with macroscopic and microscopic characters is shown in Table 1. The highest proportion was of *Aspergillus* 1842 (91.68%) followed by *Penicillium* 91(4.53%), *Mucor* 52(2.58%), *Alternaria* 7(0.35%),

Cladosporium 6(0.29%), *Fusarium* 4(0.199%) and unidentified (07%). Species of *Aspergillus* identified on the basis of colony and microscopic characters were *Aspergillus flavus, A. niger, A. parasiticus, A. ochraceous, Aspergillus carbonarious,* and *A. terreus,* shown in Figure 1. Out of 1842 *Aspergillus* isolates, the highest count was of *A. flavus* 915(49.67%) followed by *Aspergillus fumigatus* 489(26.54%), *A. niger* 373(20.24%), *A. carbonarious* 32(1.73%), *A. parasiticus* 19(1.03%), *A. ochraceous* 7(0.38%), and *A. terreus* 7(0.38%).

TABLE 1. Preliminary identification of isolated fungi along with macroscopic and microscopic characters

Sr. No.	Macroscopic characters	Microscopic characters	Tentative identification
1	Initially white turning to Black granular colony Reverse: Slightly yellowish with ridges		*Aspergillus niger*
2	Obverse: Yellowish green with cottony texture Reverse:		*Aspergillus flavus*
3	Obverse: Yellowish, rough cottony Reverse: colorless	Septate, hyaline hyphae	*Aspergillus ochraceous*
4	Obverse: brownish black, cottony to granular Reverse: colorless	Presence of vesicle covered by conidiophores,	*Aspergillus carbonarious*
5	Obverse: Dark brownish green Reverse: pale	Circular conidia arranged in chain	*Aspergillus parasiticus*
7	Obverse: Cinnamon brown Reverse: pale		*Aspergillus terreus*
8	Obverse: yellowish pale Reverse: yellowish pale		*Aspergillus fumigatus*
9	Obverse: bluish green Reverse: yellow pigment	Septate, hyaline hyphae	*Penicillium*
10	Obverse: Smoky grey, fluffy Reverse: colorless	Coenocytic, hyaline hyphae	*Mucor*
11	Obverse: pinkish cottony Reverse: slightly reddish	Septate, hyaline hyphae	*Fusarium*
12	Obverse: grayish colony, slightly wooly Reverse: black with breaks	Pigmented hyphae and spores with septations	*Alternaria*
13	Obverse: Black cottony Reverse: black and media breaks	Pigmented hyphae and spores with septations	*Cladosporium*

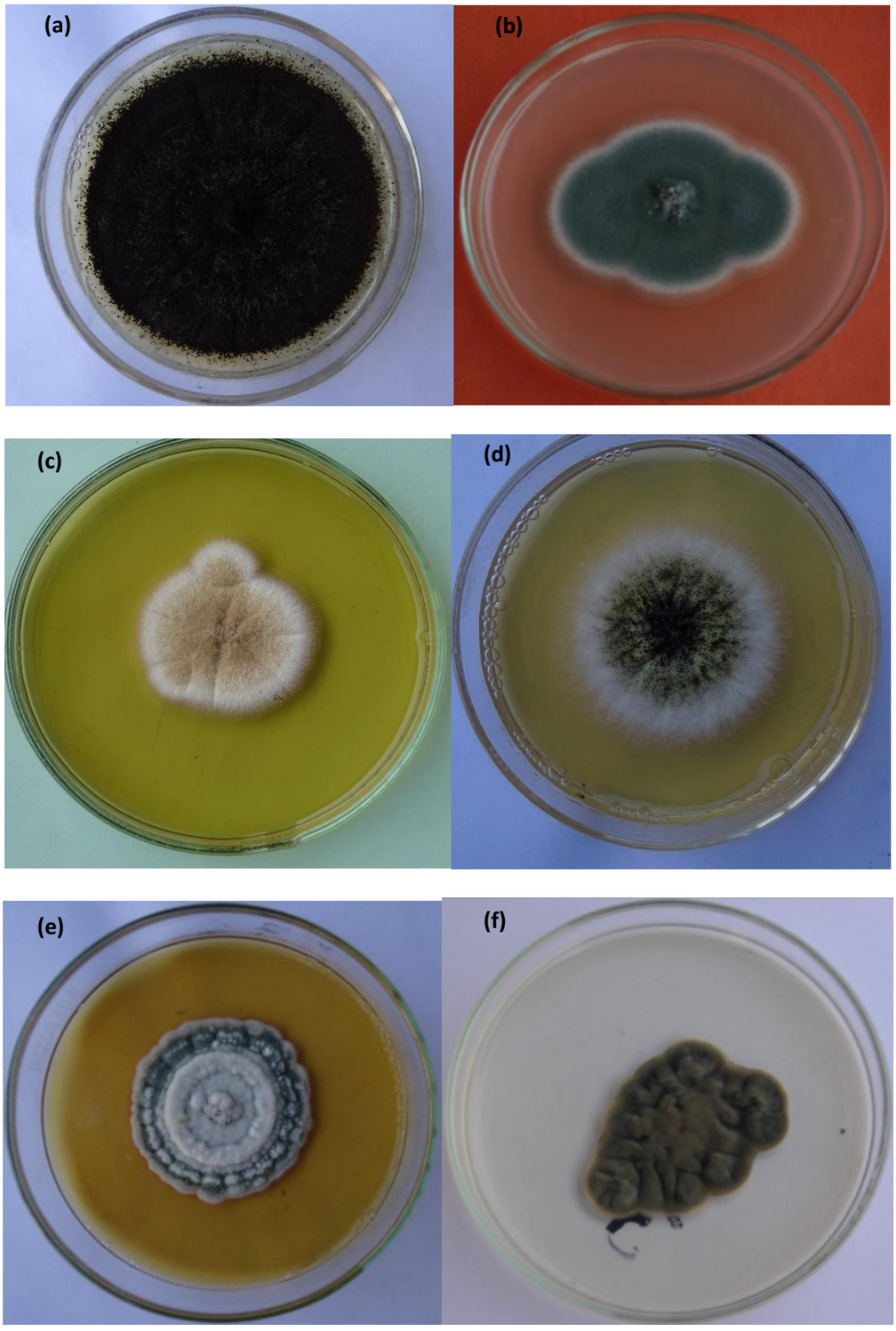

FIGURE 1. Growth morphology of differnent fungi species on Sabouraud's dextrose agar (a) *A. niger,* (b) *A. fumigatus,* (c) *A. ochraceous,* (d) *A. carbonarious,* (e) *Penicillium,* and (f) *Phaeoid fungi*

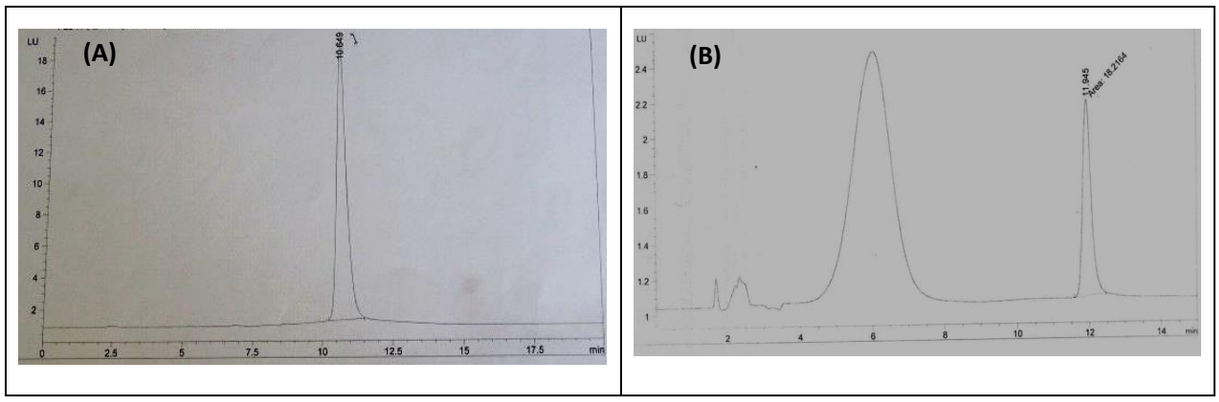

FIGURE 2. HPLC chromatograms of Ochratoxin A standard and positive sample

SELECTION OF OTA PRODUCING FUNGI

Each of the pure fungal isolate was screened for OTA production using TLC followed by HPLC and only 41 out of 1842 *Aspergillus* isolates (2.22%) were found positive. HPLC chromatograms of OTA standard and positive sample are shown in Figure 2.

MOLECULAR CHARACTERIZATION OF OCHRATOXIN PRODUCING FUNGI

OTA producing Aspergilli (n=41) were identified at specie level by PCR using specie specific primers. Amplicon size of 400 base pairs was considered positive for *Aspergillus ochraceous* having 400 bp bands, 310 bp for *A. terreus* and 430 bp for *A. parasiticus*, presented in Figure 3. Out of 41 processed *Aspergillus* isolates, 27 were identified as *A. terreus*, nine *A. parasiticus* and five *A. ochraceus.*

OTA PRODUCTION
PHYSICAL REQUIREMENTS

Confirmed OTA producing isolates (n=09) including *A. terreus* (ASPO 1-3), *A. parasiticus* (ASPO 4-6) and *A. ochraceus* (ASPO 7-9) were evaluated for toxin production potential under different physical and nutritional factors. Upon culturing at 22 ºC, the highest mean quantity of OTA (µg/mL) as determined by HPLC at pH 4.5, 6.0 and 7.5 produced was by ASPO-4 (10.86±.015 µg/mL), ASPO-4 (317.25±.10 µg/mL) and ASPO-6 (88.52±1.00 µg/mL), respectively. At 28 ºC, same pattern was observed at pH 4.5, 6.0 and 7.5, OTA produced was highest by ASPO-4 (19.79±.10 µg/mL), ASPO-4 (55.00±1.00 µg/mL) and ASPO-6 (28.10±.10 µg/mL), respectively. Similarly, at 37 ºC ASPO-4 (pH 4.5 and 6.0) and ASPO-6 (pH 7.5) produced the highest mean ochratoxin and the respective values were 257.27±.01, 968.00±10.00 and 969.45±.03 µg/mL. Overall, ASPO-6 isolate showed highest toxin production potential at pH 7.5. Graphically, optimization of OTA at different temperatures (22, 28 and 37 ºC) and pH (4.5, 6 and 7.5) is presented in Figure 4.

OTA production by selected isolates was estimated by culturing at different concentration of maize as substrate and at one percent, the isolate ASPO-4 produced highest quantity (1.38±.01 µg/mL) followed by ASPO-5 (0.38±.02 µg/mL) and least by ASPO-9 (0.04±.01 µg/mL). At concentration of 3%, the isolate ASPO-4 produced 1.48±.04 µg/mL of OTA which differed significantly with quantity of toxin produced by other isolates. At concentration of 5%, the isolate ASPO-4 produced statistically highest quantity of toxin in comparison to other cultured isolates. Overall, ASPO-4 isolate exhibited highest production potential for OTA at different used concentrations of maize in present study.

Same pattern for the production of OTA was observed while using different concentrations of wheat as substrate. Highest quantity of OTA was produced by ASPO-4 at one, three and five percent wheat as substrate which were 2.42±.02, 1.76±.20 and 1.65±.20 µg/mL, respectively. Statistically production of toxin was highest and differed significantly with the rest of the tested isolates. While using Rice (1, 3 and 5%) as substrate, same pattern for OTA was observed. Statistically highest quantity with significant difference in relation to other selected isolates was produced by ASPO-4. The quantity produced at one, three and five percent concentrations was 0.67±.01, 0.77±.05, and 0.65±.04 µg/mL, respectively. Optimizations of OTA with different substrates of varying concentration (1, 3 and 5%) are presented in Figure 5.

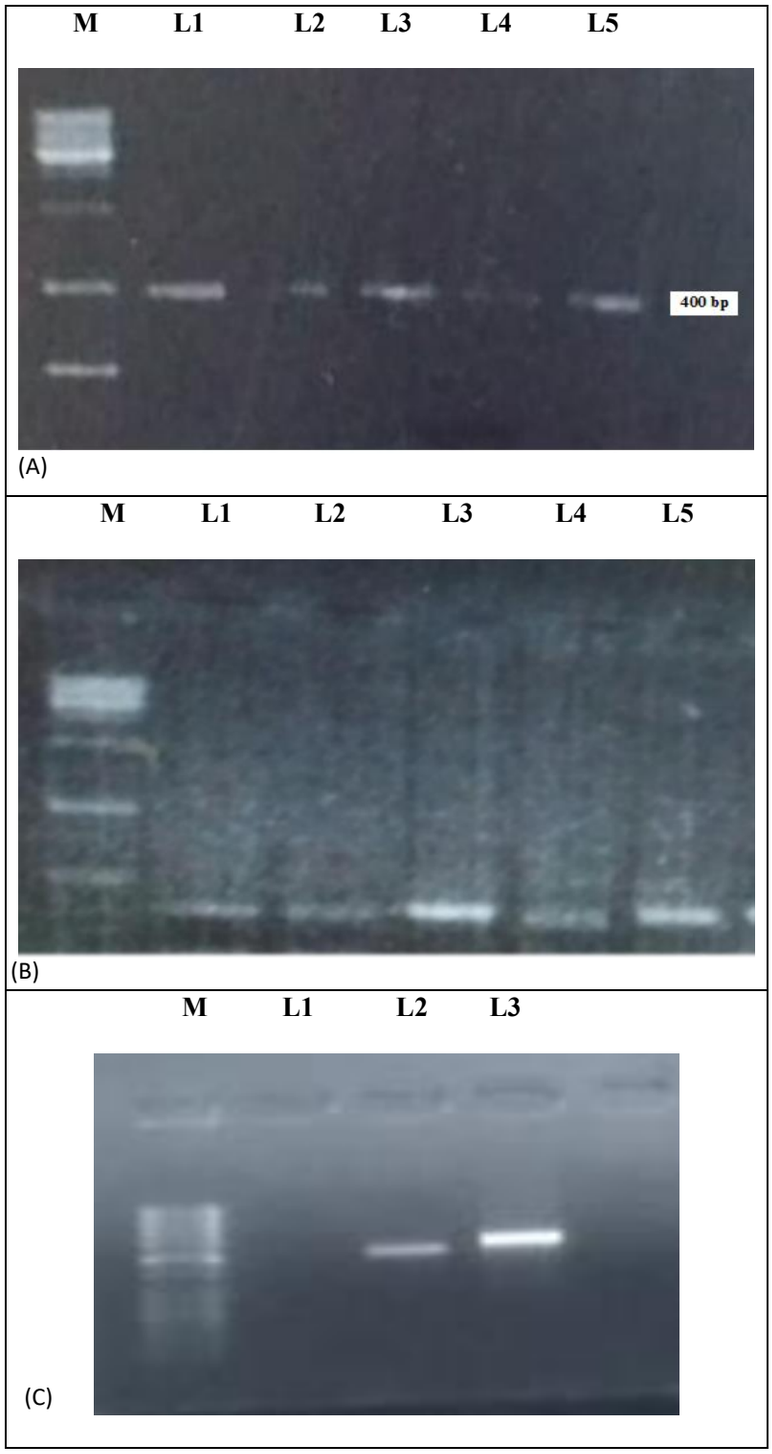

FIGURE 3. PCR images of OTA producing *Aspergillus* species (A) Lane M: 100bp DNA marker; Lane 1-5: *Aspergillus ochraceous* isolates 1 to 5 (400-bp). (B) Lane M: DNA marker; Lane1 to 5: *Aspergillus terreus* isolates 1-5 (310-bp). (C) Lane M: DNA marker; Lane 2-3: *Aspergillus parasiticus* isolates 1 to 2 (430-bp)

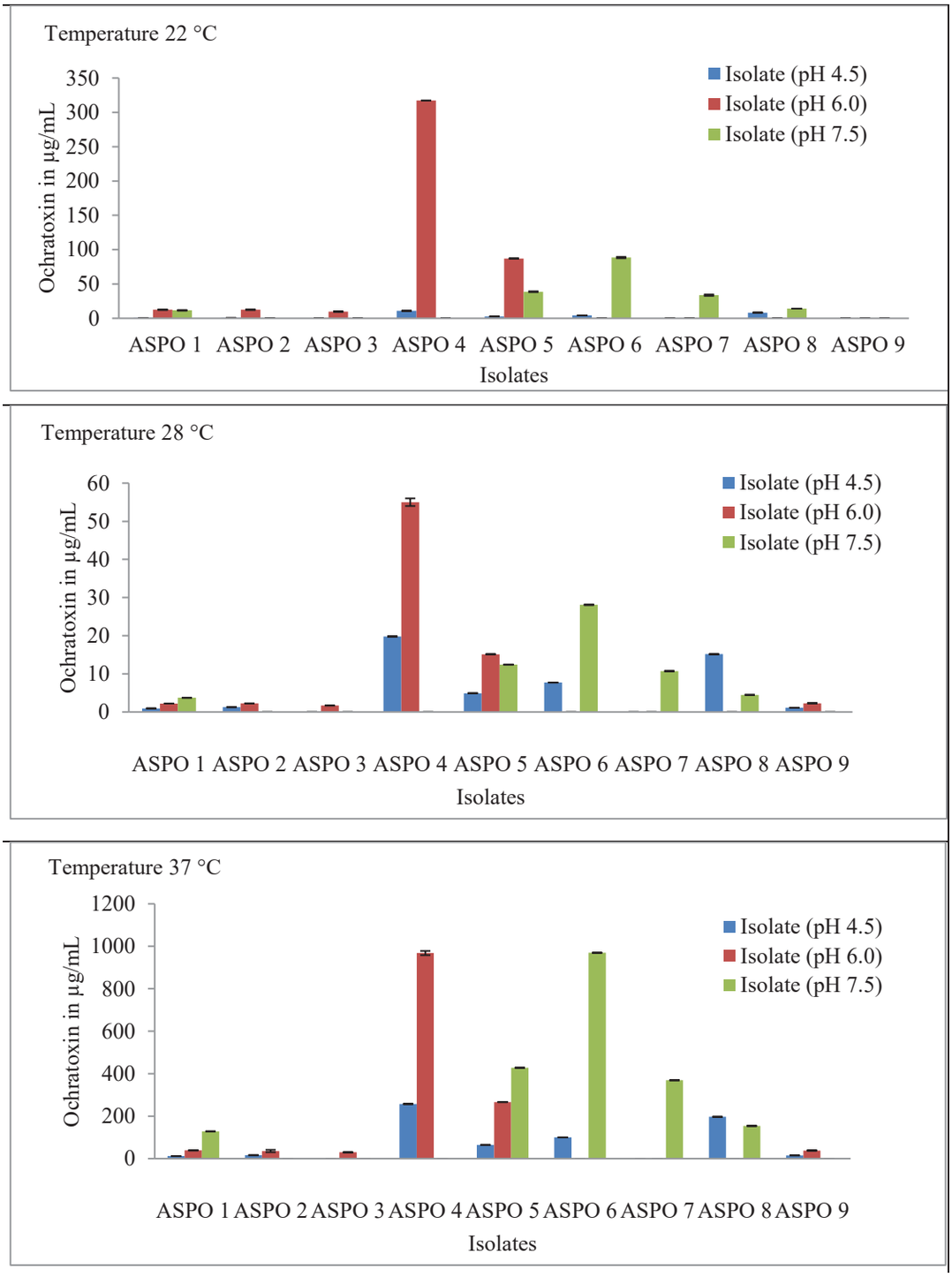

FIGURE 4. Optimization of OTA at different temperatures (22, 28 and 37 °C) and pH (4.5, 6 and 7.5)

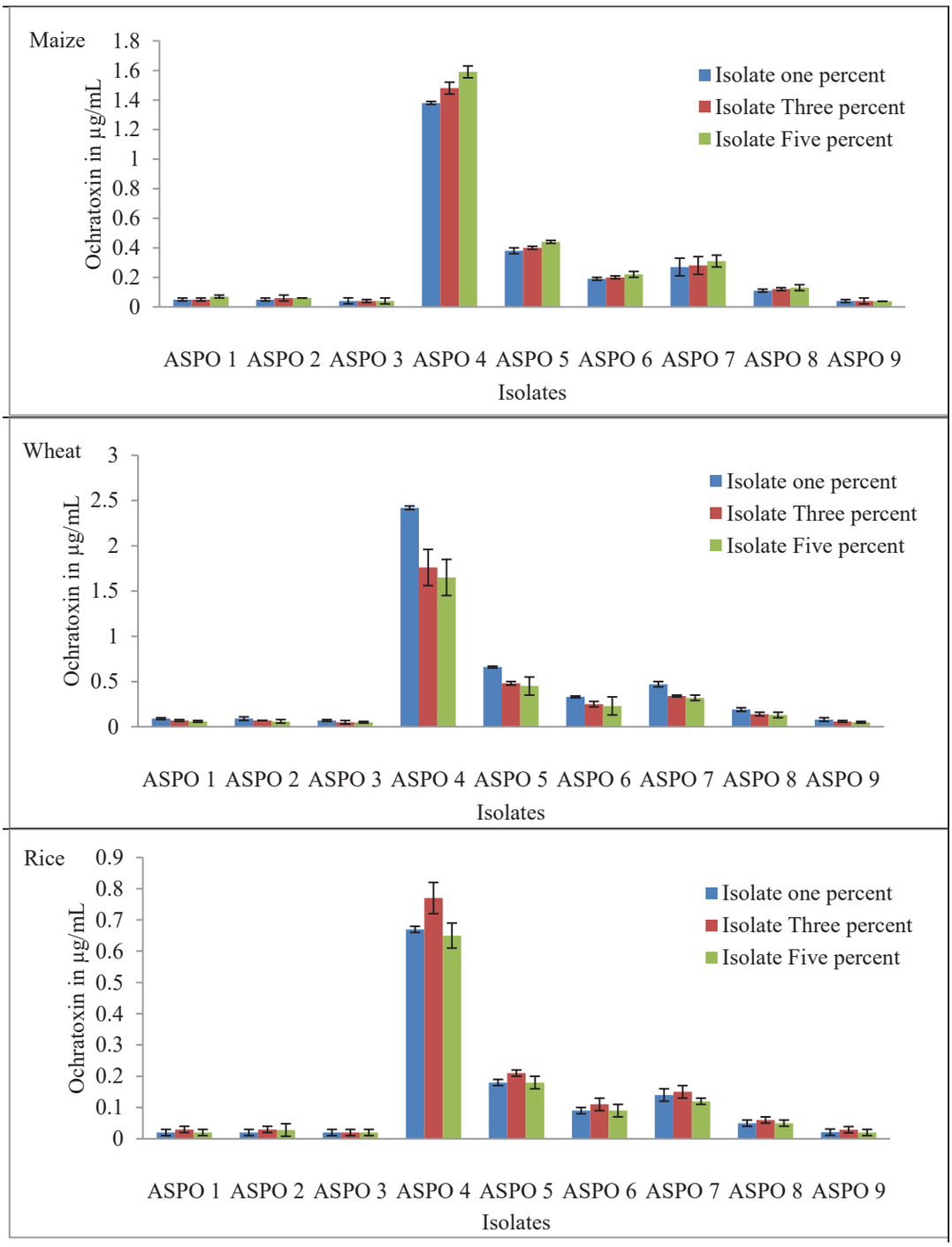

FIGURE 5. Optimization of OTA with different substrates of varying concentration (1, 3 and 5%)

STABILITY EVALUATION OF OTA

For stability profile, OTA standards (100 µg/mL and 100 µg/g) were prepared by using three organic solvents (chloroform, acetonitrile and methanol) and three sterilized organic solids (egg yolk, skimmed milk powder and sucrose). Standards were evaluated after two months, four months and six months. Percentage log reduction was calculated and the highest percentage reduction (100%) was shown on storage in most of the storage vials. After six months, least reduction in OTA was calculated in chloroform placed in Opaque colored vial and Sucrose placed in Opaque and transparent vials (shown in Table 2). Highest reduction of OTA was observed in skimmed milk and egg yolk within all three kinds of vials. Least reduction was in OTA stored in chloroform (opaque vial) and sucrose (transparent and opaque vials). OTA showed more stability in chloroform and sucrose.

TABLE 2. Percentage reduction of Ochratoxin A under different storage conditions

Standard type	02 Month (µg/mL)	04 Month (µg/mL)	06 Month (µg/mL)
Methanol (T)	18.47	56.32	86.69
Methanol (B)	99.12	100	100
Methanol (O)	71.46	98.88	100
Acetonitrile (T)	71.76	98.66	100
Acetonitrile (B)	49.91	51.957	98.91
Acetonitrile (O)	98.97	100	100
Chloroform (T)	97.22	100	100
Chloroform (B)	82.53	97.05	98.05
Chloroform (O)	0	0	0
Egg Yolk (T)	44.96	100	100
Egg Yolk (B)	43.22	80.05	100
Egg Yolk (O)	100	100	100
Sucrose (T)	0	0	0
Sucrose (B)	70.88	96.59	100
Sucrose (O)	0	0	0
Skimmed Milk (T)	83.68	100	100
Skimmed Milk (B)	23.88	53.41	100
Skimmed Milk (O)	100	100	100

DISCUSSION

In present study, isolates of different genera including *Aspergillus, Penicillium, Mucor, Alternaria, Cladosporium* and *Fusarium* recovered from poultry feed. The highest proportion was of *Aspergillus* 1842(91.68%) followed by *Penicillium* 91(4.53%), *Mucor* 52(2.58), *Alternaria* 7(0.35%), *Cladosporium* 6(0.29%), *Fusarium* 4(0.199%) and unidentified (07) in accordance

with the study conducted by Nwiyi et al. (2019). Five fungal isolates were recovered from poultry feed includes *Aspergillus, Penicillium, Fusarium, Mucor* and yeast from all the feed samples. *Aspergillus* (87%) recorded as highest percentage, followed by *Penicillium* (27%), *Fusarium* (24%), yeast (5%) and *Mucor* (2%), respectively (Nwiyi et al. 2019). In another study main fungal genus found in corn-based poultry feed were *Aspergillus* (43.1%), *Fusarium* (52.9%) and *Penicillium* spp. (41.2%).

Aspergillus and *Penicillium* were abundantly present in feed samples while *Fusarium* was the predominantly isolated genus from the corn samples (Nasaruddin et al. 2021). The most frequent fungal genus found were *Fusarium* spp. (90%) and *Aspergillus* spp. (70%) in mashed as well as pelleted feed, respectively. The most frequent recovered genus in pelleted feed was *Aspergillus* while the most frequent genus in mashed feed were *Fusarium* and *Aspergillus* (Ghaemmaghami & Nowroozi 2018). The fungi isolated from poultry feed samples were *A. niger*, *Penicillium* spp., *Fusarium* spp., *Rhizopus* spp., *Mucor* spp., and *Cladosporium* spp. (Danbappa et al. 2018). The genus *Fusarium* was the most prominent isolated from corn (45.1%), soybean meal (30.5%) before pelleting (61.2%), while *Aspergillus* genus was the predominantly present in samples of pellets (29.1%). Totally, the most frequent fungus isolated from ingredients and finished feed was *Fusarium* (36.4%), followed by *Aspergillus* (21.8%), *Penicillium* (11.4%) and yeast (11.4%) (Ghaemmaghami et al. 2016). The most frequently isolated genera of fungi from fish feed were *Aspergillus* (86.66%) followed by *Penicillium* (23.33%), *Fusarium* spp. (10%), *Mucor* (6.66%), and *Rhizopus* (3.33%) somewhat relevant to our study (Kholife et al. 2019).

Species of *Aspergillus* identified in present study were *A. flavus, A. niger, A. parasiticus, A. ochraceous, A. carbonarious,* and *A. terreus.* The highest count was of *A. flavus* (49.67%) followed by *A. fumigatus* (26.54%), *A. niger* (20.24%), *A. carbonarious* (1.73%), *A. parasiticus* (1.03%), *A. ochraceous* (0.38%), and *A. terreus* 7(0.38%). These results are in line with a previous study on poultry feed showed that the highest percentage was of *A. flavus* (95%) followed by *A. niger* (75%), *A. fumigatus* (15%) and *A. terreus* (5%) (Sana et al. 2019). The most frequent isolates from feed ingredients and finished feed were *A. flavus* (64%), *A. niger* (16.6%) followed by *A. fumigatus* (10.7%), *A. ochraceus* (2.4%), and *Aspergillus glaucus* (2.4%) (Ghaemmaghami et al. 2016) somewhat related to present study. In another study the isolation frequency of *Aspergillus* species as follows: *A. flavus* (47%), *A. niger* (24%), *A. fumigatus* (17%), *Aspergillus oryzae* (3%), *Aspergillus tamarii* (2%) closely related to our study (Aboagye-Nuamah, Kwoseh & Maier 2021). *A. niger* was most prevalent (43.33%) followed by *A. flavus* (30%), *A. fumigatus* (6.66%), *Aspergillus versicolor* (3.33%), and *A. terreus* (3.33%) in fish feed (Kholife et al. 2019). A. *flavus* was the most frequently isolated (42%), *A. fumigatus*, (9%) *A. parasiticus* (3%), *Aspergillus nidulans* (5%), *A. niger* (1.67), *A. terreus* (1%), and *Aspergillus nomius* (0.33%) (Ibrahim et al. 2017) in agreement with the present study.

In the current study, each of the pure fungal isolate was screened for OTA production using TLC followed by HPLC and only 2.22% *Aspergillus* isolates were found positive for OTA. In a recent study, an automated on-line two-dimensional high-performance liquid chromatography method (2D-HPLC) is used to determine OTA in different food samples (Armutcu, Uzun & Denizli 2018). The OTA was quantified in another study by a high-performance liquid chromatography (HPLC-FD) with a limit of detection (LOD) of 0.05 ng/mL for OTA (Moez et al. 2020). In a recent study OTA was detected in wine by high-performance liquid chromatography (Freire et al. 2017) in line with our study.

In the current study, OTA producing *Aspergilli* were identified at specie level by PCR using specie specific primers. Out of 41 processed *Aspergillus* isolates, 27 were identified as *A. terreus*, nine *A. parasiticus* and five *A. ochraceus* as OTA producer. In contrast to our study, four isolates of *A. niger* and *Aspergillus tubingensis* were reported as OTA producer characterized by molecular along with phylogenetic analyses from poultry feed in Iran (Gherbawy, El-Dawy & Mohamed 2019). Furthermore, OTA production in various fungal species isolated from poultry feed were evaluated and it was high in *P. verrucosum* (5.5 µg/kg) and *A. niger* (1.1 µg/kg). Sixty three *A. niger* S15KS primer pairs (Gherbawy, El-Dawy & Mohamed 2019). In another study, *pks* gene was detected in four strains isolates were OTA producers showing the presence of *pks* genes by using PKS15C-MeT and PKf *A. ochraceus* (10%) obtained from sausage samples and then sequenced (Algammal et al. 2021) somewhat related to our study. Another study based on specie specific primers, *A. ochraceus* isolates presented the key genes for ochratoxigenic (*pks*) biosynthesis pathways (Hassan et al. 2018).

The confirmed OTA producing isolates were evaluated in this study for toxin production potential under different physical and nutritional factors including temperature, pH and different substrates. Upon culturing at 22 °C, 28 °C and 37 °C, the highest mean quantity of OTA (µg/mL) as determined by HPLC at pH 4.5, 6.0 and 7.5 produced was by *A. ochraceus, A. terreus,* and *A. parasiticus* isolates. In this research study, ASPO-4 (*A. parasiticus*) is able to produce high OTA in wheat, maize and white rice and overall, ASPO-6 (*A. parasiticus*) showed the highest toxin production at pH 7.5. In a recent study, biomass production of two strains of *A. carbonarious* increased proportionally with time and reached a maximum at 25 °C and 0.94 a_w and at day 3 no growth present in both strains at 0.94 a_w despite of temperature and also at 0.98 a_w with temperature of 20 °C (Lappa et al. 2017). In another study, the conditions under which strains show the highest OTA risk index are a_w between 0.95 and 0.99 and temperatures between 22 and 32 °C for *A. carbonarious* strains and a_w between 0.97 and 0.99 and temperatures between 25 and 30 °C for *A. ochraceus* stains (Oliveira et al. 2019). In another study, mycelium growth of *A. ochraceus* was inhibited under pH 4.5 and 10.0, while the sporulation increased under alkaline condition (Wang et al. 2018). In another study, after 6 days incubation, maximum fungal growth was detected with no significant difference in the mycelia growth of the three *A. carbonarious* at the different levels of pH (Lasram et al. 2012) and these results are in line with our study.

For stability profile in this study, OTA standards (100 µg/mL and 100 µg/g) were prepared by using three organic solvents (chloroform, acetonitrile, and methanol) and three sterilized organic solids (egg yolk, skimmed milk powder, and sucrose). It was shown that the highest percentage reduction (100%) was on storage in most of the storage vials. Highest reduction of OTA was observed in skimmed milk and egg yolk within all three kinds of vials. Least reduction was in OTA stored in chloroform (opaque vial) and sucrose (transparent and opaque vials). OTA showed more stability in chloroform and sucrose. In a recent study it is observed that OTA is stable in methanol for at least 50 days when stored at about 5 °C. The stability of OTA in methanol and methanol-water-acetic acid for standard solutions by HPLC-FLD or -MS. They were stable when kept at about 5 °C and OTA concentration was constant for at least 50 days storage (Kiseleva et al. 2020). Liazid et al. (2007) studied stability of OTA in methanol (~50 µg/mL) and OTA concentration was determined by HPLC-FLD. Shaking of solution at temperatures 25 to 60 °C for at least 20 min with or without microwave assisted extraction at temperatures up to 150 °C for 20 min have not led to any significant change in OTA concentration. There is no sufficient data still available for stability profile of OTA in solids such as skimmed milk and egg yolk. The current stability study helps in long term storage of OTA standards in different solvents as well solids.

Conclusion

Ochratoxin contamination in food and feed is a serious health risk for animals and humans. Poultry feed ingredients have been reported to be contaminated with OTA particularly when stored for long a period of time. Isolates of different genera were recovered from poultry feed and pure fungal isolate was screened for OTA production. Also confirmed OTA producing isolates were evaluated for toxin production potential under different physical and nutritional factors, prepare the OTA and performed the stability studies as well. Continuous surveillance should be conducted to detect OTA contaminated cereals and contamination level in poultry feed. Good agricultural management practice should also be employed to reduce contamination risk of OTA and OTA producing fungi in agricultural commodities.

Acknowledgements

The authors are grateful to Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan, for conducting this research work.

References

Aboagye-Nuamah, F., Kwoseh, C.K. & Maier, D.E. 2021. Toxigenic mycoflora, aflatoxin and fumonisin contamination of poultry feeds in ghana. *Toxicon* 198(2): 164-170.

Algammal, A.M., Elsayed, M.E., Hashem, H.R., Ramadan, H., Sheraba, N.S., El-Diasty, E.M., Abbas, S.M. & Hetta, H.F. 2021. Molecular and HPLC-based approaches for detection of aflatoxin B 1 and ochratoxin A released from toxigenic Aspergillus species in processed meat. *BMC Microbiology* 21(1): 1-10.

Antonio-Bautista, A., Vazquez-Badillo, M., Galindo-Cepeda, M.E., Flores-Naveda, A., Benítez-Neri, E., Sánchez-Arizpe, A. & Arispe-Vázquez, J. 2021. Effect of the temperature and relative humidity in stored sotol (Dasylirion cedrosanum Trel.) seeds on fungi biodiversity. *Journal of Phytology* 13: 16-20.

Armutcu, C., Uzun, L. & Denizli, A. 2018. Determination of Ochratoxin A traces in foodstuffs: Comparison of an automated on-line two-dimensional high-performance liquid chromatography and off-line immunoaffinity-high-performance liquid chromatography system. *Journal of Chromatography A* 1569: 139-148.

Becker, P., Normand, A.C., Vanantwerpen, G., Vanrobaeys, M., Haesendonck, R., Vercammen, F., Stubbe, D., Piarroux, R. & Hendrickx, M. 2019. Identification of fungal isolates by MALDI-TOF mass spectrometry in veterinary practice: Validation of a web application. *Journal of Veterinary Diagnostic Investigation* 31(3): 471-474.

Bhat, R., Rai, R.V. & Karim, A.A. 2010. Mycotoxins in food and feed: Present status and future concerns. *Comprehensive Reviews in Food Science and Food Safety* 9(1): 57-81.

Cervini, C., Verheecke-Vaessen, C., Ferrara, M., García-Cela, E., Magistà, D., Medina, A., Gallo, A., Magan, N. & Perrone, G. 2021. Interacting climate change factors (CO_2 and temperature cycles) effects on growth, secondary metabolite gene expression and phenotypic ochratoxin A production by *Aspergillus carbonarius* strains on a grape-based matrix. *Fungal Biology* 125(2): 115-122.

Danbappa, A.A.R., Alhassan, K.A. & Shah, M.M. 2018. Isolation and identification of microbial contaminants associated with commercial poultry feeds. *Journal of Applied and Advanced Research* 3(5): 142-147.

Dudakova, A., Spiess, B., Tangwattanachuleeporn, M., Sasse, C., Buchheidt, D., Weig, M., Groß, U. & Bader, O. 2017. Molecular tools for the detection and deduction of azole antifungal drug resistance phenotypes in Aspergillus species. *Clinical Microbiology Reviews* 30(4): 1065-1091.

Ehrlich, K.C., Kobbeman, K., Montalbano, B.G. & Cotty, P.J. 2007. Aflatoxin-producing Aspergillus species from Thailand. *International Journal of Food Microbiology* 114(2): 153-159.

Freire, L., Passamani, F.R.F., Thomas, A.B., Nassur, R.D.C.M.R., Silva, L.M., Paschoal, F.N., Pereira, G.E., Prado, G. & Batista, L.R. 2017. Influence of physical and chemical characteristics of wine grapes on the incidence of *Penicillium* and *Aspergillus* fungi in grapes and ochratoxin A in wines. *International Journal of Food Microbiology* 241: 181-190.

Gaur, A. & Adholeya, A. 2000. Effects of the particle size of soil-less substrates upon AM fungus inoculum production. *Mycorrhiza* 10(1): 43-48.

Ghaemmaghami, S.S., Modirsaneii, M., Khosravi, A.R. & Razzaghi-Abyaneh, M. 2016. Study on mycoflora of poultry feed ingredients and finished feed in Iran. *Iranian Journal of Microbiology* 8(1): 47.

Ghaemmaghami, S.S. & Nowroozi, H. 2018. Toxigenic fungal contamination for assessment of poultry feeds: Mashed vs. Pellet. *Iranian Journal of Toxicology* 12(5): 5-10.

Gherbawy, Y.A., El-Dawy, E.G. & Mohamed, H.M. 2019. Molecular characterization of ochratoxin A-producing fungi isolated from poultry feed. *Journal of Experimental and Applied Animal Sciences* 3(1): 15-27.

Greco, M.V., Franchi, M.L., Golba, S.L.R., Pardo, A.G. & Pose, G.N. 2014. Mycotoxins and mycotoxigenic fungi in poultry feed for food-producing animals. *The Scientific World Journal* 2014: 1-9.

Hassan, Z.U., Al-Thani, R.F., Migheli, Q. & Jaoua, S. 2018. Detection of toxigenic mycobiota and mycotoxins in cereal feed market. *Food Control* 84: 389-394.

Hendrix, J.D., Zhang, X., Campbell, Y.L., Zhang, L., Siberio, L., Cord, C.L., Silva, J.L., Goddard, J., Kim, T., Phillips, T.W. & Schilling, M.W. 2018. Effects of temperature, relative humidity, and protective netting on *Tyrophagus putrescentiae* (Schrank) (Sarcoptiformes: Acaridae) infestation, fungal growth, and product quality of dry cured hams. *Journal of Stored Products Research* 77: 211-218.

Henry, T., Iwen, P.C. & Hinrichs, S.H. 2000. Identification of Aspergillus species using internal transcribed spacer regions 1 and 2. *Journal of Clinical Microbiology* 38: 1510-1515.

Hussaini, A.M., Timothy, A.G., Olufunmilayo, H.A., Ezekiel, A.S. & Godwin, H.O. 2009. Fungi and some mycotoxins found in mouldy Sorghum in Niger State, Nigeria. *World Journal of Agricultural Sciences* 5(1): 5-17.

IARC. 1993. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* 56. Geneva: WHO.

Ibrahim, M., Kabir, J., Kwanashie, C., Salawudeen, M. & Joshua, Z. 2017. Occurrence of mycotoxigenic fungi in poultry feeds at live-bird markets, Zaria, Nigeria. *Sokoto Journal of Veterinary Sciences* 15: 53-59.

Illueca, F., Vila-Donat, P., Calpe, J., Luz, C., Meca, G. & Quiles, J.M. 2021. Antifungal activity of biocontrol agents *in vitro* and potential application to reduce mycotoxins (Aflatoxin B1 and Ochratoxin A). *Toxins* 13(11): 752.

Kehinde, M., Oluwafemi, F., Itoandon, E., Orji, F. & Ajayi, O. 2014. Fungal profile and aflatoxin contamination in poultry feeds sold in Abeokuta, Ogun State, Nigeria. *Nigerian Food Journal* 32(1): 73-79.

Kholife, M., Moawad, A., Diab, A.M. & El-keredy, A. 2019. Mycological examination of fish feed stuff with special reference to mycotoxin production. *Slovenian Veterinary Research* 56(22): 303-312.

Kiseleva, M., Chalyy, Z., Sedova, I. & Aksenov, I. 2020. Stability of mycotoxins in individual stock and multi-analyte standard solutions. *Toxins* 12(2): 94.

Koh, C.Y., Modahl, C.M., Kulkarni, N. & Kini, R.M. 2018. Toxins are an excellent source of therapeutic agents against cardiovascular diseases. *Seminars in Thrombosis and Hemostasis* 6: 1-15.

Lappa, I.K., Kizis, D. & Panagou, E.Z. 2017. Monitoring the temporal expression of genes involved in ochratoxin a production of *Aspergillus carbonarius* under the influence of temperature and water activity. *Toxins* 9(10): 296.

Lasram, S., Barketi, A., Mliki, A. & Ghorbel, A. 2012. Growth and ochratoxin A production by *Aspergillus carbonarius* at different pHs and grape maturation stages. *Letters in Applied Microbiology* 54(5): 418-424.

Liazid, A., Palma, M., Brigui, J. & Barroso, C.G. 2007. Investigation on Ochratoxin A stability using different extraction techniques. *Talanta* 71(2): 976-980.

Maciorowski, K., Herrera, P., Jones, F., Pillai, S. & Ricke, S. 2007. Effects on poultry and livestock of feed contamination with bacteria and fungi. *Animal Feed Science and Technology* 133(1-2): 109-136.

Mantle, P. 2002. Risk assessment and the importance of ochratoxins. *International Biodeterioration & Biodegradation* 50(3-4): 143-146.

Moez, E., Noel, D., Brice, S., Benjamin, G., Pascaline, A. & Didier, M. 2020. Aptamer assisted ultrafiltration cleanup with high performance liquid chromatography-fluorescence detector for the determination of OTA in green coffee. *Food Chemistry* 310: 125851.

Nasaruddin, N., Jinap, S., Samsudin, N.I., Kamarulzaman, N.H. & Sanny, M. 2021. Prevalence of mycotoxigenic fungi and assessment of aflatoxin contamination: A multiple case study along the integrated corn-based poultry feed supply chain in Malaysia. *Journal of the Science of Food and Agriculture* 101(5): 1812-1821.

Nwiyi, P.O., Nwabuko, C.N., Ndubueze, A. & Christain, O. 2019. Isolation and identification of mycotoxigenic organisms in poultry feed from selected locations in Abia State, Nigeria. *Asian Journal of Research in Animal and Veterinary Sciences* 3(4): 1-9.

Oliveira, G., Evangelista, S.R., Passamani, F.R.F., Santiago, W.D., Cardoso, M. das-G. & Batista, L.R. 2019. Influence of temperature and water activity on Ochratoxin A production by Aspergillus strain in coffee south of Minas Gerais/Brazil. *Lebensmittel-Wissenschaft & Technologie* 102: 1-7.

Pardo, E., Marín, S., Ramos, A. & Sanchis, V. 2005. Effect of water activity and temperature on mycelial growth and ochratoxin A production by isolates of *Aspergillus ochraceus* on irradiated green coffee beans. *Journal of Food Protection* 68(1): 133-138.

Pena, A., Cerejo, F., Lino, C. & Silveira, I. 2005. Determination of ochratoxin A in Portuguese rice samples by high performance liquid chromatography with fluorescence detection. *Analytical and Bioanalytical Chemistry* 382(5): 1288-1293.

Petzinger, E. & Weidenbach, A. 2002. Mycotoxins in the food chain: the role of ochratoxins. *Livestock Production Science* 76(3): 245-250.

Pleadin, J. 2015. Mycotoxins in grains and feed: Contamination and toxic effect in animals. *Biotechnology in Animal Husbandry* 31(4): 441-456.

Rafique, A., Javed, M. & Ali, S.S. 2018. Contamination of commercial poultry feeds and feed ingredients with aspergillus flavus and aflatoxin b1 in punjab pakistan. *Journal of Animal and Plant Sciences* 28(4): 1021-1026.

Ren, P., Ahearn, D. & Crow, S.J. 1999. Comparative study of Aspergillus mycotoxin production on enriched media and construction material. *Journal of Industrial Microbiology and Biotechnology* 23(3): 209-213.

Rosa, C.D.R., Palacios, V., Combina, M., Fraga, M., Rekson, A.D.O., Magnoli, C. & Dalcero, A.M. 2002. Potential ochratoxin A producers from wine grapes in Argentina and Brazil. *Food Additives & Contaminants* 19(4): 408-414.

Saddozai, A.A., Raza, S. & Saleem, S.A. 2012. Microbial count and shelf life of strawberry juice. *Pakistan Journal of Agricultural Research* 25(3): 218-223.

Saleemi, M.K., Khan, M.Z., Khan, A. & Javed, I. 2010. Mycoflora of poultry feeds and mycotoxins producing potential of Aspergillus species. *Pakistan Journal of Botany* 42(1): 427-434.

Sana, S., Anjum, A.A., Yaqub, T., Nasir, M., Ali, M.A. & Abbas, M. 2019. Molecular approaches for characterization of aflatoxin producing *Aspergillus flavus* isolates from poultry feed. *Pakistan Veterinary Journal* 39(2): 169-174.

Sivakumar, V., Singaravelu, G. & Sivamani, P. 2014. Isolation, characterization and growth optimization of toxicogenic molds from different animal feeds in Tamilnadu. *International Journal of Current Microbiology and Applied Sciences* 3(9): 430-445.

Trail, F., Mahanti, N. & Linz, J. 1995. Molecular biology of aflatoxin biosynthesis. *Microbiology* 141(4): 755-765.

Wang, Y., Liu, F., Wang, L., Wang, Q., Selvaraj, J.N., Zhao, Y., Wang, Y., Xing, F. & Liu, Y. 2018. pH-signaling transcription factor AopacC regulates ochratoxin A biosynthesis in *Aspergillus ochraceus*. *Journal of Agricultural and Food Chemistry* 66(17): 4394-4401.

Zeng, D., Lin, Z., Zeng, Z., Fang, B., Li, M., Cheng, Y-H. & Sun, Y. 2019. Assessing global human exposure to T-2 toxin via poultry meat consumption using a lifetime physiologically based pharmacokinetic model. *Journal of Agricultural and Food Chemistry* 67(5): 1563-1571.

*Corresponding author; email: aftab.anjum@uvas.edu.pk